

## Effect of Parenteral Immunization on the Intestinal Immune Response to *Salmonella typhi* Ty21a

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The effects of parenteral administration of a killed typhoid vaccine on the intestinal immune response to live orally administered *Salmonella typhi* Ty21a in human subjects was evaluated. Priming with parenteral vaccination neither enhanced nor suppressed the subsequent specific serum and intestinal immunoglobulin A (IgA) immune responses to a booster course of live oral vaccine. Neither a single oral dose of live vaccine nor a single dose of parenteral vaccine had any measurable booster effect on the observed primary intestinal IgA response to the live oral vaccine. Two booster doses of subcutaneously administered killed typhoid vaccine did result in a significant increase in the specific intestinal IgA antibody in those subjects primed with the oral live vaccine. This response was comparable in magnitude to the primary intestinal response. No evidence of this response could be found in serum IgA, although nonsignificant rises in serum IgG were evident. Previous parenteral priming had no effect on secondary immune responses to a live oral vaccine in humans. Serum immune responses were generally found to be of little value as indicators of local intestinal immunity. This study confirmed that parenteral vaccination was only able to induce an intestinal immune response following priming with live, orally administered organisms and that multiple parenteral booster doses were necessary to induce a measurable effect on intestinal immune responses.

It has been long held that the optimal means of inducing a mucosal immune response has been through the local rather than systemic administration of a vaccine or antigen (15, 23). This has been reinforced through the persistent failure of parenterally administered vaccines alone to consistently stimulate mucosal immunity in nonimmune animals or humans (20, 24, 25, 28, 30).

However, there are examples in which the efficient induction of mucosal immune responses by using combined systemic-local vaccination schedules has occurred (5, 16, 27, 28). Equally, there are reports which have shown that systemic immunization may actually suppress mucosal secretory immunoglobulin A (sIgA) responses (14, 26, 29). It has been suggested that these contradictory observations may reflect deficiencies either in the design of these respective studies, particularly in technical considerations such as the failure to adequately characterize and define the antigen dose-response curve or the time course of the desired sIgA response, or in the ability to repeatedly evoke the response (26).

The parenterally administered heat-killed typhoid vaccine has been extensively used by travellers and for supporting public health control measures in the prevention of typhoid fever for many years. Recently, the live oral typhoid vaccine *Salmonella typhi* Ty21a has become widely available as the accepted prophylaxis against typhoid fever. Since the heat-killed parenterally administered typhoid vaccine at best only afforded short-term protection, it would appear likely that there are a considerable number of individuals who have been previously vaccinated with this preparation who are likely to be revaccinated orally with *S. typhi* Ty21a.

The following studies were designed to ascertain the effects of parenteral vaccination on the specific intestinal immune responses induced by the live orally administered typhoid vaccine organism *S. typhi* Ty21a in human subjects. In particular, two questions that may affect the widespread use of the respective vaccine formulations needed to be answered: whether previous parenteral vaccination with killed typhoid vaccine in naive subjects prevents the effective induction of an intestinal immune response by orally administered *S. typhi* Ty21a and whether parenteral vaccination with killed typhoid vaccine potentiates the local intestinal immune response in naive subjects following the oral administration of *S. typhi* Ty21a.

### MATERIALS AND METHODS

**Subjects.** Thirty healthy adults (9 women and 21 men, 18 to 33 years of age) agreed to participate in this study. Written and informed consent was obtained from all subjects prior to their entry into the study. The use of human subjects was in accordance with the ethical standards of the Human Ethics Committee of the Royal Adelaide Hospital and the Committee on the Ethics of Human Experimentation of the University of Adelaide and with the Helsinki Declaration of 1975. None of the subjects had any previous exposure to typhoid fever, through vaccination or disease. None of the subjects had any history or current symptoms of gastrointestinal tract disease.

The subjects were allocated randomly to five study groups, groups A to E. Each group was vaccinated by means of a route, dose, formulation, and schedule as detailed in Table 1.

**Vaccine preparations.** *S. typhi* Ty21a is an attenuated Vi antigen-negative mutant of the pathogenic strain *S. typhi* Ty2 (12, 34). We have extensive experience in the use of this organism in our experimental human model, having accu-

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TABLE 1. Subject groups, vaccine doses,<sup>a</sup> and sampling schedules<sup>b</sup>

Group (no. of subjects)	Primary vaccination		Booster vaccinations		Timing of intestinal intubations (days) <sup>d</sup>
	Route	Schedule (days) <sup>c</sup>	Route	Schedule (days) <sup>c</sup>	
A (7)	Oral	0, 2, 5			15, 29, 43
B (6)	Oral	0, 2, 5	Oral	21	21, 42
C (5)	Oral	0, 2, 5	SC	21	21, 42
D (6)	Oral	0, 2, 5	SC	29, 57	22, 50, 78
E (6)	SC	0, 16	Oral	31, 33, 36	31, 52

<sup>a</sup> Oral doses comprised a mean of  $1.7 \times 10^{11}$  viable vaccine organisms; subcutaneous (SC) doses comprised a mean of  $5 \times 10^8$  killed organisms.

<sup>b</sup> Serum samples were obtained from all subjects prevaccination and then every 3 or 4 days from the commencement of vaccination and every 3 or 4 days following the booster vaccinations.

<sup>c</sup> Days from commencement of vaccination.

<sup>d</sup> All subjects underwent prevaccination intestinal intubation the week before commencement of vaccination. Values are timing of postvaccination intestinal intubations in days after commencement of vaccination.

rately defined its dose-response curve (8) and repeatability of induction of mucosal sIgA responses for a given dose (1, 6–9) and having documented the time course of that response previously (6, 8).

All *S. typhi* Ty21a vaccine doses used in this study were supplied by Enterovax Limited, Salisbury, South Australia, Australia, as individual lyophilized doses. Each dose comprised a mean total number of organisms by direct microscopy of  $5.2 \times 10^{11}$ , with  $1.7 \times 10^{11}$  (33%) viable by colony counts. This vaccine dose was selected since we have previously demonstrated with previously unexposed subjects that three sequential doses of  $10^9$  live *S. typhi* Ty21a organisms in a liquid suspension failed to stimulate a measurable intestinal immune response, while the enteric-coated capsule formulation, which we have shown previously to contain  $10^9$  viable organisms and at least  $5 \times 10^{10}$  killed *S. typhi* Ty21a organisms, induced a meager intestinal immune response largely attributable to the killed organisms present (8). We and others have shown previously that doses of  $10^{11}$  viable organisms consistently stimulate a significant specific intestinal IgA antibody response without noticeable adverse effects when administered to volunteer subjects (1, 2, 6–9, 12).

Each dose of *S. typhi* Ty21a vaccine was orally administered according to the vaccination schedule detailed in Table 1. Subjects were required to fast for 8 h prior to vaccination; the vaccine organisms were swallowed following the ingestion of 50 ml of a 2% sodium bicarbonate solution. This pretreatment was necessary to neutralize gastric acid of subjects. Gastric acid has been demonstrated to have an adverse effect on the viability of live orally administered enteric organisms (11) as well as being able to alter the immunogenicity of inactivated oral vaccine preparations (4). Five minutes following this pretreatment, these subjects ingested the vaccine dose which had been previously suspended in 40 ml of 0.9% saline for 15 min at room temperature and was followed by 100 ml of distilled water.

The parenterally administered typhoid vaccine used in groups C, D, and E was the commercially available heat-killed preparation (Typhoid Vaccine; Commonwealth Serum Laboratories, Parkville, Victoria, Australia); 0.5 ml ( $5 \times 10^8$  organisms) was administered subcutaneously by injection.

The priming course of *S. typhi* Ty21a used for subject groups A to D was the standard three-dose schedule de-

scribed above. In group E, all subjects received the killed typhoid vaccine according to the minimum schedule recommended by the vaccine manufacturer, a single dose at least 2 weeks apart (days 0 and 16), with the oral booster schedule commencing 15 days later (day 31). In both groups B and C the booster dose was administered on day 21. However, because of scheduling difficulties involved in using healthy human volunteer subjects for long-term studies, the first parenteral booster dose for group D could not be administered until day 29.

**Collection of samples.** Intestinal fluid samples were obtained from the upper jejunum with an ANPRO AN20 Andersen tungsten-weighted sump tube (H. W. Andersen Products, Oyster Bay, N.Y.) according to the schedule detailed in Table 1. In all subjects, correct positioning of the intestinal tube was confirmed by using fluoroscopy. The use of fluoroscopy for this purpose was restricted by the Ethics Committees, limiting the number of times the procedure could be performed according to previous radiation exposure, usually a maximum of four intubations. Sampling occurred between 15 and 21 days following the commencement of oral vaccination, since this time has been shown to represent the timing of the peak response after primary vaccination (8). Intestinal sampling after parenteral vaccination occurred either 16 days after the completion of the primary vaccination course or 16 to 21 days following each postprimary booster dose (Table 1).

Intestinal fluid samples with a pH of  $>6.5$  were collected and kept on ice until 25 ml had been collected from each subject. This technique of intestinal intubation has proven to be quite effective for obtaining suitable samples of intestinal fluid for the determination of specific sIgA levels (1–3, 6–10, 18, 19). The samples were centrifuged at  $4,000 \times g$  at  $4^\circ\text{C}$  and stored at  $-70^\circ\text{C}$  until required. The timing of the intestinal intubations was intended to detect the maximal immune responses after vaccination (8). It has been our experience that long-term storage of untreated intestinal fluid at or below  $-70^\circ\text{C}$  does not adversely affect the specific or total immunoglobulin levels (7).

Serum samples were collected before and every 3 or 4 days after vaccination until the completion of the study and stored as 1-ml portions at  $-20^\circ\text{C}$ .

**ELISA for quantifying specific antibody.** Class-specific anti-typhoid antibodies in serum and intestinal secretions were quantified by using a previously described enzyme-linked immunosorbent assay (ELISA) (6). Briefly, 96-well polyvinyl microtiter ELISA plates (Costar catalog number 2595; Data Packaging Corp., Cambridge, Mass.) were coated with *S. typhi* Ty2 lipopolysaccharide (Sigma Chemical Co., St. Louis, Mo.; catalog number L 6386) and blocked with 0.05% (wt/vol) bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Serum diluted 1:10 and intestinal fluid diluted 1:2 in BSA-PBS obtained from individual subjects were added to duplicate wells on the plates. Subsequently, the serum and intestinal fluid samples were sequentially diluted 1:3 and 1:2, respectively, in BSA-PBS down the plate, giving eight dilutions. The plates were incubated at  $37^\circ\text{C}$  for 16 h. After a wash, alkaline-phosphatase-conjugated goat anti-human IgA, IgG, or IgM antisera (KPL; Kirkegaard and Perry Laboratories, Gaithersburg, Md.) were added, and the plates were incubated at  $37^\circ\text{C}$  for 4 h. After a wash, 0.100 ml of a 1-mg/ml solution of the substrate *p*-nitrophenylphosphate (phosphatase substrate 104-105; Sigma) in a 10% (vol/vol) diethanolamine buffer was added to all wells, and after further incubation at  $37^\circ\text{C}$  for 2 h, the

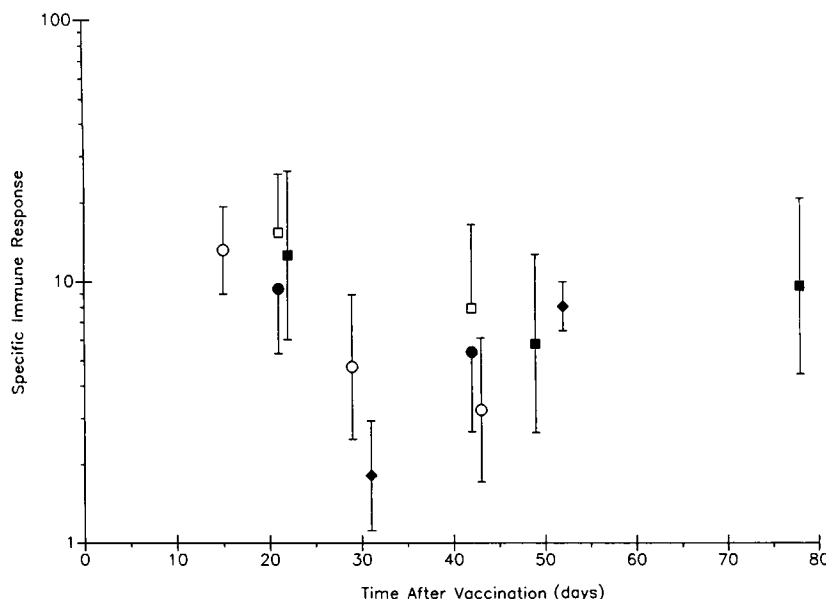


FIG. 1. Effects of parenteral immunization on specific intestinal IgA responses to oral vaccine *S. typhi* Ty21a. The specific intestinal antibody response is given as the geometric mean fold rise in postvaccination specific IgA antibody titer over prevaccination titer adjusted for total IgA ( $\pm 95\%$  confidence intervals).  $\circ$ , group A (oral, no boost);  $\bullet$ , group B (oral, oral);  $\square$ , group C (oral, subcutaneous);  $\blacksquare$ , group D (oral, subcutaneous, subcutaneous);  $\blacklozenge$ , group E (subcutaneous, subcutaneous, oral). Vaccination doses and schedules are detailed in Table 1.

plates were read with a Titertek ELISA reader, model 310C, at 405 nm.

In each assay, serum obtained from a convalescent typhoid patient with a known high antibody titer directed against *S. typhi* lipopolysaccharide was included as a positive control, and serum obtained from an unexposed individual with a known low antibody titer against *S. typhi* lipopolysaccharide was included as a negative control.

Serum and intestinal antibody responses were calculated as units of antigen-specific antibody, defined as the reciprocal of the dilution that gave an optical density of 0.15 ELISA absorbance units/0.100 ml (the volume added to each well). This absorbance was chosen as it represented the upper limit of the 95% confidence intervals above background levels (32). The intestinal fluid-specific antibody units were adjusted for total IgA content to give antibody responses as units of specific antibody per milligram of total IgA. Both serum and intestinal antigen-specific antibody responses are expressed as the fold rise in postvaccination antibody units over the levels of the prevaccination sample.

**Radial immunodiffusion assay for quantifying total intestinal immunoglobulin.** A single radial immunodiffusion method was used to determine the total class-specific immunoglobulin content of intestinal fluid (22). A glass plate was coated with a 1% agarose-sodium barbitone solution containing rabbit anti-human IgA ( $\alpha$ -chain specific) immunoglobulin (catalog number ORCI 14/15; Behringwerke AG, Marburg, Germany) to a final concentration of 0.026 mg/ml. Intestinal fluid samples were applied neat, with 0.003 ml of each sample being added in duplicate to the wells of the plate. The standard curve for the determination of total IgA in intestinal fluid was constructed by using sIgA in the form of human colostrum of predetermined IgA and sIgA content (2).

The plate was stored in a humid atmosphere at 4°C for 72 h and then washed four times in 0.9% (wt/vol) saline, each wash lasting 12 h, after which it was rinsed with distilled water to remove any salt residue and then dried in a 56°C

oven until the agarose was hard. The dried plate was stained with a 0.1% (wt/vol) solution of Coomassie blue dissolved in a water-acetic acid-methanol (50:7:50) solution, with excess stain being removed by rinsing the plates with a double-distilled water-acetic acid-methanol (85:7:5) solution.

The immunoglobulin concentration of any particular sample was determined by comparison to the diameters of precipitation rings formed by the standards of known concentration on the same plate.

**Statistics.** The significance of any differences between the postvaccination mean fold rises of three or more different sample groups were determined by the one way analysis of variance for independent samples. Comparisons of the fold rises in specific antibody titers between two groups were analyzed by using the nonparametric two-tailed Wilcoxon rank sum test for independent data.

## RESULTS

**Intestinal IgA-specific antibody response.** From Fig. 1 it is evident that oral vaccination with the three alternate-day doses of  $10^{11}$  *S. typhi* Ty21a gave rise to a significant increase in postprimary vaccination intestinal anti-typhoid specific IgA response which did not differ significantly between any of the subject groups A to D. This was expected from our previous observations (6–9). It was clear from examination of the day 42-day 43 (groups A, B, and C) and day 49 (group D) samples in Fig. 1 that none of the subsequent booster doses, either as a single oral dose of  $10^{11}$  viable organisms (group B) or as a single subcutaneous dose of killed vaccine organisms (groups C and D) significantly altered the decline in specific anti-typhoid IgA antibody from that of the control group, group A ( $P = 0.14$ ).

It was only after the second subcutaneously administered killed typhoid vaccine dose (group D, day 78) that there was evidence of an effect with the significant enhancement in the local intestinal antibody response compared with that mea-

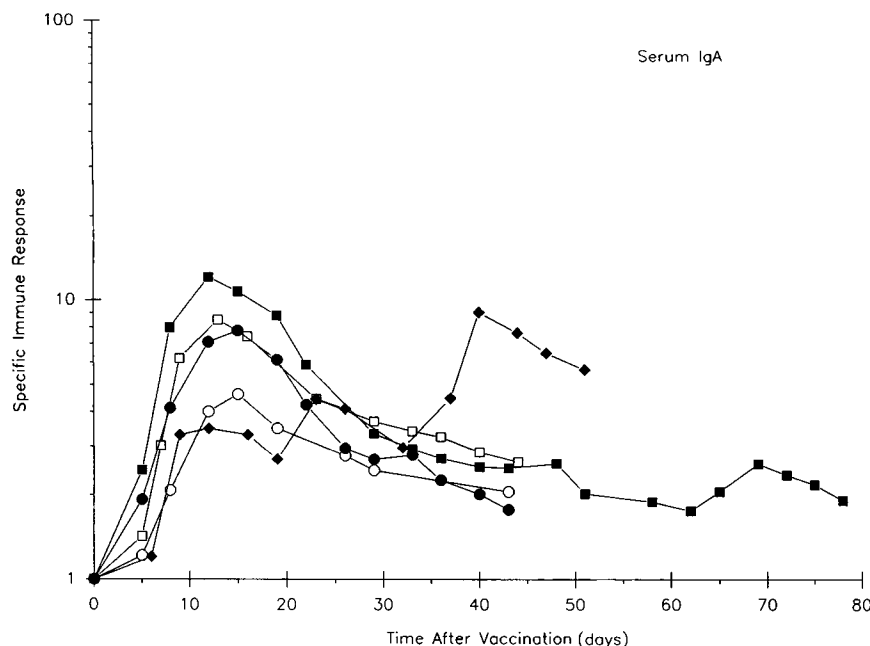


FIG. 2. Effects of parenteral immunization on specific serum IgA responses to oral vaccine *S. typhi* Ty21a. Specific serum antibody response is given as the geometric mean fold rise in postvaccination specific IgA antibody titer over prevaccination titer. See the legend to Fig. 1 for symbol definitions.

sured in the last sample (day 43) obtained from the control (nonboosted) group, group A ( $P = 0.014$ ). This response did not differ in magnitude from that observed after the primary oral immunization ( $P = 0.31$ ) but did represent a significant increase in the mean fold rise in specific IgA antibody over that observed after the first subcutaneously administered booster dose (day 49,  $P = 0.031$ ).

Primary vaccination by subcutaneous administration of two doses of killed *S. typhi* vaccine organisms (group E) resulted in a weak nonsignificant intestinal anti-typhoid IgA-specific antibody response. The group E booster course, comprising the three alternate daily orally administered doses of  $10^{11}$  *S. typhi* Ty21a, resulted in an intestinal specific IgA antibody response that was not significantly different from the intestinal immune response observed in the groups receiving the three oral doses as a primary vaccination ( $P = 0.14$ ).

We have reported previously that intestinal IgG and IgM responses are erratic and frequently undetectable following oral vaccination with *S. typhi* Ty21a (6). This was again our experience in this study, so these results have been omitted.

**Serum IgA-specific antibody response.** The serum anti-typhoid IgA-specific antibody responses are depicted in Fig. 2. No significant difference between the mean fold rises in the postvaccination specific IgA antibody responses after the primary vaccination with either the three alternate daily live oral doses or the two subcutaneously administered killed doses was apparent ( $P = 0.36$ ). The decline in specific IgA antibody following the peak primary immune response in the orally vaccinated groups (groups A, B, C, and D) occurred irrespective of the nature of the booster dose, such that there was no significant difference between the mean fold rises of the day 43-day 44 serum samples ( $P = 0.34$ ). Unexpectedly, no significant enhancement of specific serum IgA was observed after the second booster dose of subcutaneously administered killed typhoid vaccine (group D) compared with that of the last control (group A) sample ( $P > 0.50$ ).

Parenteral priming with two subcutaneously administered killed typhoid vaccine doses (group E) did not appear to impair the specific immune response to a subsequent three alternate daily dose course of live oral typhoid vaccine. The specific IgA response after the booster course did not appear to differ significantly from the responses observed in groups A, B, C, and D, who received the live vaccine by using that course as a primary vaccination ( $P = 0.087$ ).

**Serum IgG-specific antibody response.** Despite the perceived differences in the magnitude of the primary immune responses evident in groups A, B, C, and D in Fig. 3 after the three doses of the live oral typhoid vaccine, the individual variability of the responses is reflected in the observation that these differences are not statistically significant ( $P = 0.31$ ). Furthermore, these primary responses did not differ from that induced by the first of the two subcutaneous doses of killed typhoid vaccine (group E;  $P = 0.20$ ).

Neither the single oral (group B) nor the single subcutaneous (group C) booster doses after a primary live oral vaccination course appeared to significantly influence the kinetics of the IgG-specific antibody response compared with that in the control group, group A. In group D, each subcutaneous booster dose resulted in a small specific IgG antibody peak (Fig. 3). However, these vaccine doses appeared to be of little long-term benefit, since after the final booster dose there is significant decline in specific antibody from the final peak on day 62 to day 78 ( $P = 0.050$ ).

The parenterally administered killed vaccine (group E) induced a specific IgG antibody response of shorter duration than observed with the three-dose live oral vaccination schedule, yet its use for the induction of a primary immune response appeared to have no obvious effect on the secondary immune response to an orally administered vaccine course (Fig. 3). The postboosting secondary specific IgG antibody response of group E was not significantly different from that observed after the oral vaccine was used to induce

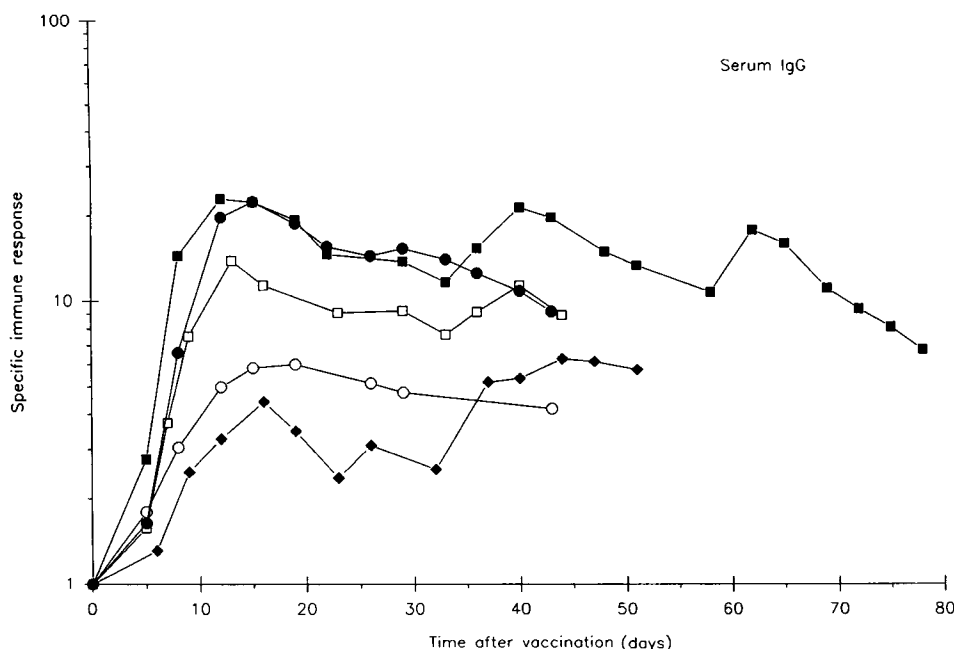


FIG. 3. Effects of parenteral immunization on specific serum IgG responses to oral vaccine *S. typhi* Ty21a. Specific serum antibody response is given as the geometric mean fold rise in postvaccination specific IgG antibody titer over prevaccination titer. See the legend to Fig. 1 for symbol definitions.

a primary immune response as seen in groups A, B, C, and D ( $P = 0.34$ ).

**Serum IgM-specific antibody response.** We have reported previously that serum IgM responses have proven unhelpful in documenting the immune response to intestinally presented antigens in the human small intestine (6, 8). The IgM responses again proved too variable and of too low a magnitude to be of value in this study.

## DISCUSSION

The studies reported here have investigated the effect of parenterally administered killed typhoid vaccine on the induction of a humoral mucosal immune response to an orally administered live typhoid vaccine, *S. typhi* Ty21a, in human subjects.

Parenteral priming did not appear to have any describable effect on the intestinal immune response to the orally administered *S. typhi* Ty21a, inducing neither suppression nor enhancement of the response. The secondary oral vaccination course induced an intestinal immune response indistinguishable from that seen in subjects receiving a similar primary oral vaccination course. This is an important finding, since it implies that the effectiveness of oral bacterial vaccines should not be influenced by previous parenteral vaccination status.

This finding differs from observations in rats intraperitoneally immunized with cholera toxin in which subsequent immune responses to intrajejunal cholera toxin administration were suppressed (17), although it is consistent with responses to cholera toxin in rabbit Thiry-Vella loops (36). Our contrasting results may possibly be explained by fundamental differences in the mechanisms involved in the induction of mucosal immune responses by soluble (e.g., protein) antigens compared with particulate antigens (37).

Neither a single subcutaneous booster dose of killed

typhoid vaccine nor an oral booster dose of live *S. typhi* Ty21a after priming with a short course of live oral *S. typhi* Ty21a enhanced the local intestinal immune response; neither had any obvious effect on the decline in specific IgA antibody from that observed in the unboosted controls. The failure of the first parenteral booster dose to increase the intestinal immune response may reflect suboptimal sampling time or weakness of the secondary response. The failure of the single oral vaccine dose to boost the primary immune responses is most likely an example of immune exclusion, as oral vaccination occurred in the presence of an activated local immune system as reflected in good, albeit declining, specific intestinal IgA antibody levels.

Further parenteral boosting with killed vaccine administered 2 weeks after the first dose was observed to induce a significant enhancement in intestinal typhoid specific IgA antibody which was not reflected in any significant change in specific serum IgA or IgG antibody. Others have observed that in lactating Pakistani women, administration of either a single or two sequential subcutaneous doses of killed cholera vaccine in the presence of low levels of anti-cholera antibody induced significant rises in cholera specific IgA antibody in saliva and breast milk (13, 31). In neither case was intestinal antibody activity measured.

One possible explanation for our observations could be that systemically distributed antigen stimulates intestinal antibody-secreting cells. This may either invoke an enhanced local production of antigen-specific immunoglobulin or stimulate a mucosal memory response. That such a response is not evident after a single boosting dose may suggest that the quantity of antigen distributed in this manner is quite low and that an enhanced response is only evident after repeated exposures.

This study may also offer an explanation for the observed protective efficacy of parenterally administered killed vaccines when evaluated in areas in which the disease is

endemic, because previous mucosal exposure is likely to result in enhanced local immunity upon subsequent exposure to the antigen, even when presented systemically. This should caution against the extrapolation of conclusions concerning the protective efficacy of such vaccines to non-endemic areas.

The contribution of specific local immunoglobulin to protection against typhoid fever in humans remains uncertain. On the single occasion in which *S. typhi* Ty21a was assessed in previously unexposed human subjects, a protective efficacy of 87% against clinical typhoid fever was demonstrated (12). This protection, achieved by using multiple oral vaccine doses containing  $3 \times 10^{10}$  to  $1 \times 10^{11}$  viable organisms, could not be correlated to specific intestinal antibody, since such determination was not performed on that occasion. All subsequent evaluations of *S. typhi* Ty21a have occurred in typhoid endemic areas utilizing doses of  $10^9$  viable organisms (21, 33–35), with the resulting protection rates most likely reflecting boosting of preexisting naturally acquired specific immunity. Within the limits of our study design it is clear that after a good primary local immune response, which in the immunologically naive subject can only be achieved with doses exceeding  $10^{10}$  viable organisms (8), two further parenteral vaccinations with killed vaccine organisms can potentiate the local immune response well beyond that achieved by the primary oral vaccination course. However, the contribution of this prolongation of specific local antibody response to protection against typhoid remains uncertain and warrants further investigation.

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